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14. ABSTRACT Virtually all chemotherapy agents, at clinically achievable concentrations, act by inducing cancer cell death via apoptosis, but cancer cells eventually become resistant to apoptosis-inducing therapeutic agents. Necrosis, with fewer mechanisms of resistance, might be exploited specifically against prostate cancer cells by induction of "targeted necrosis". Targeted necrosis has potential clinical utility, since its cell death mechanism retains the cancer cell specificity of apoptosis and bypasses apoptotic resistance by re-direction into necrosis. Previous studies found that p53p-Ant, a p53 C-terminal peptide fused at its C-terminus to the carrier peptide of antennapedia, induced rapid apoptosis via activation of the Fas/FADD/caspase-8 pathway. In these studies we found synergistic targeted necrosis in prostate cancer cells pre-incubated with paclitaxel, after treatment with p53p-Ant. Paclitaxel treatment alone induced transcription (RT-PCR) and translation (western blotting) of Fas ligand. Addition of dominant negative FADD enhanced necrosis by p53p-Ant, but repressed the synergistic activity with paclitaxel, suggesting the involvement of FAS/FADD pathway. Previous studies indicated that p53p-Ant activates the Fas receptor by flipping the receptor in the extracellular membrane making it available for activation by ligand. Therefore, pre-treatment with paclitaxel may prime the cells by induction of Fas ligand so that subsequent exposure to p53p-Ant efficiently activates the Fas/FADD cell death pathway.

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Introduction

By understanding the mechanisms and pathways of targeted necrosis, as it differs from classical apoptosis, may help to elucidate how we can manipulate available drugs and p53 peptides for inducing this alternate pathway in human prostate cancer cells to circumvent resistance to classical apoptosis. This may lead to a novel therapeutic approach, which overcomes the resistance to apoptosis which occurs in prostate cancer when exposed to taxanes which are the major chemotherapy drug class useful against this cancer. Previous studies found that p53p-Ant, a p53 C-terminal peptide fused at its C-terminus to the carrier peptide of antennapedia, induced rapid apoptosis via activation of the Fas/FADD/caspase-8 pathway. Additionally, this peptide was more toxic to human cancer cells with a mutant p53 phenotype than a wild-type p53 phenotype due to higher binding affinity to mutant p53 as compared to the wild-type p53. We found that p53p-Ant induced rapid targeted necrosis in prostate cancer cells, and was found to be dependent on mutant p53 and mediated by O2-.

CHAPTER 1: Examination of Caspases in p53p-Ant-induced targeted necrosis in prostate cancer

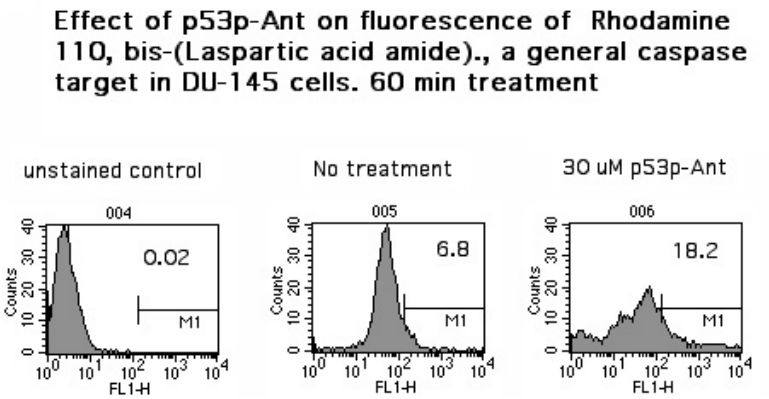
SOW Aim1, #4 #11.

Apoptosis can be switched to necrosis when cells are exposed to an apoptotic stimulus but are deprived of glucose (1) or ATP, or if PARP is not inactivated by caspases such as caspase 3 or 7 (2-4). If PARP is not inactivated, the ADP and eventually ATP pools decrease until necrosis becomes the dominant pathway. Thus, caspase inhibitors have been used to switch apoptotic cells to necrosis (2). Our previous studies have shown that the major capsases involved in Fas-mediated apoptosis, such as caspase 3, caspase 8 and caspase 9 were not activated in DU-145 cells after treatment with p53p-Ant. (5). However, this does not rule out additional capsases which may be activated and involved in p53-pAnt induced targeted necrosis. An analysis to determine if caspases in general may be induced upon treatment of prostate cancer cells with p53p-Ant is shown in Fig. 1. There was clearly activation of fluorescent rhodamine 110, which becomes fluorescent green in response to general caspase activation, indicating that there may be caspase activation after p53p-Ant treatment. Since unknown caspases may be involved in p53p-Ant-induced targeted necrosis, we tested various inhibitors together with p53p-Ant. Inhibition of caspases together with p53p-Ant may inhibit cell death or potentially increase targeted necrosis. Increasing targeted necrosis may also be useful in converting cells that normally undergo apoptosis to targeted necrosis to overcome apoptotic resistance.

The effect of the caspase inhibitor Z-DEVD-FMK, specific for caspases 3 and 7, had little effect on p53p-Ant induced targeted necrosis and one experiment is shown (Fig. 2). Another inhibitor of caspases in addition to other enzymes is Disulfiram (DSF, Antabuse). DSF has been clinically used as an alcohol deterrent, due to its ability to inhibit aldehyde dehydrogenase (6). Previous clinical use of this drug for the past 30 years makes it particularly attractive. DSF at $\geq$5 μM concentrations (Clinically achievable concentrations) also inhibits caspases (7) such as caspase 3 and 7 which could prevent the cleavage of PARP by these caspases, triggering necrosis. Active, non-cleaved PARP could lower ADP/ATP pools, which would favor the depleted ATP milieu for switching apoptosis to necrosis. As a single agent, DSF has been shown to induce apoptosis *in*

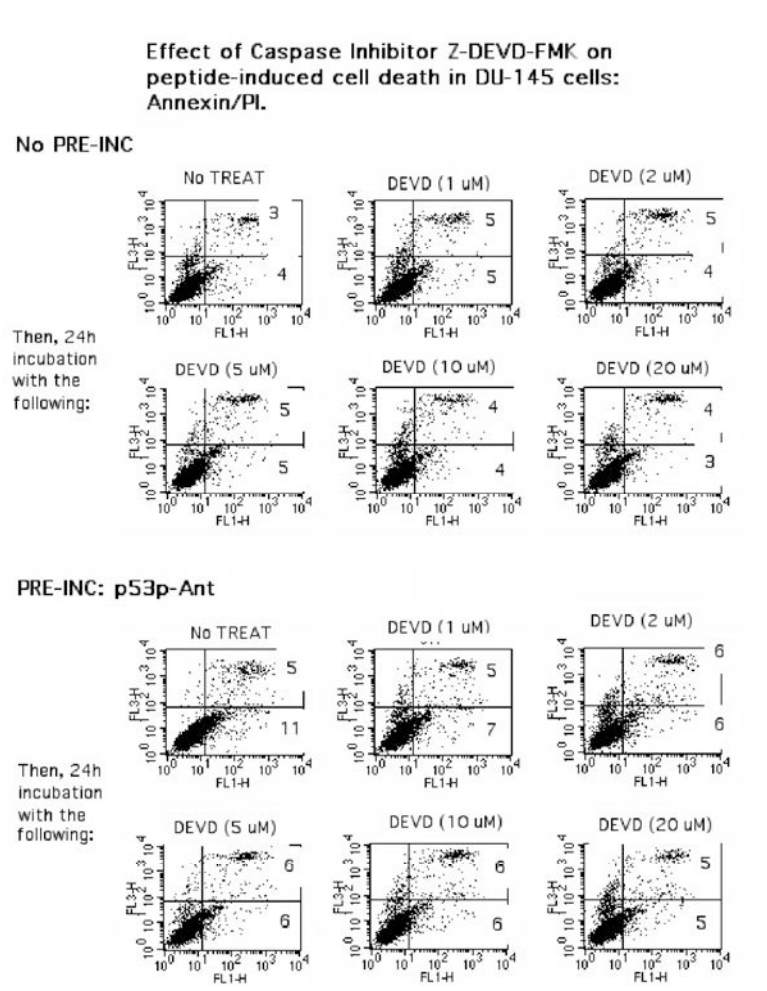
vitro in human melanoma cells associated with depleted pools of GSH (8, 9), inhibit human melanoma tumor growth in mice(9), and permanently inactivate the human MDR1 P-glycoprotein (P-gp) membrane pump (10). DSF has been shown to lower GSH through its potent oxidation of protein thiols such as GSH (7). Prostate cancer (PC) cells frequently overexpress P-gp and it is thought to be one of its important and frequent clinical mechanisms of drug resistance in over 40% of PC patients (11). DSF contains a reactive disulfide bond, which efficiently reacts with intracellular proteins and low molecular weight thiols forming disulfides, mixed disufides, and dithiocarbamates. Caspases (7, 12), particularly caspase-3 and 7 which cleave PARP, topoisomerase I and II (13) and protein kinase C isozymes (14) have been shown to directly interact with disulfiram forming protein mixed disulfides leading to caspase inactivation. We found that incubation with DSF after pre-treatment with p53p-Ant greatly enhanced the ability of p53-Ant to induce necrosis in prostate cancer cells (Fig. 3). Although DSF itself had no effect on necrosis (Control=5%, DSF= 3%), and at sub-optimal concentrations of 20 uM, p53p-Ant also had little effect (9%), incubation with as little as 0.5 uM DSF resulted in 24% necrosis (Fig. 3). This effect was seen only if cells were incubated with DSF second, after exposure to p53p-Ant rather than exposure first to DSF. (Fig. 4). Curiously, the post-treatment with DSF also converted cells to a more apoptotic, less necrotic phenotype after a 24 hour incubation period. This is further shown and corroborated in Fig 5. Also, incubation with β-mercaptoethanol, which breaks S-H bonds, disrupted the effect of DSF on p53p-Ant death (Fig 5), indicating that DSF may be forming disulfide links with a protein in the targeted necrosis pathway. As mentioned, we also observed that if the DU-145 PC cells were incubated with DSF for a period of time after exposure to p53p-Ant at low concentrations (<20 uM) for a brief period, that the cells become Annexin+ and less PI+. This may indicate a possible route to understand the switch point between the commitment to undergo necrosis and the development of apoptosis.

Figure 1:



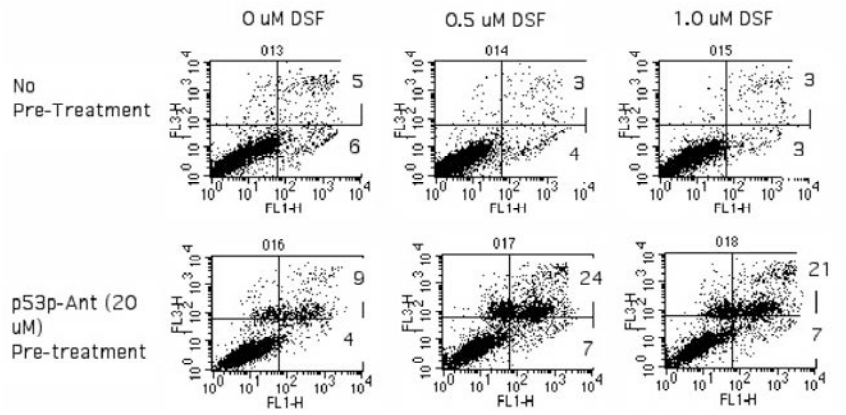
Rhodamine 110 caspase substrates are bisamide derivatives of the fluorochrome in which peptides are covalently linked to the amino groups of rhodamine. Upon enzymatic cleavage, the bisamide substrate is converted into the R110 cleavage product with spectral qualities similar to fluorescein, which can be detected in the FL-1 channel on flow cytometric analysis. The substrate used was aspartic acid, which is the most general of the available substrates. Other substrates available are based on target peptide sequences for different groups of caspases.

Figure 2:



Flow cytometric analysis of Annexin/PI positive cells either pre-incubated with p53p-Ant followed by treatment with Z-DEVD-FMK for an additional 24h. There was little effect on cell death up to 20 μM Z-DEVD-FMK. Therefore, inhibition of caspase 3 does not synergize with p53p-Ant induced cell death in DU-145 under these conditions. See Fig. 3 for similar conditions using DSF for positive control.

Figure 3: Effect DSF on p53p-Ant-induced necrosis.



Flow cytometric analysis of Annexin/PI positive cells either pre-incubated with p53p-Ant followed by treatment with DSF for an additional 24h. There was a synergistic effect of Annexin+/PI+ cells observed from 9-24% o from 9-21% with no effect of DSF alone. Therefore, p53p-Ant or DSF alone at these concentrations had little or no effect on cell death, but p53p-Ant followed by DSF treatment had a greater than 4 fold increase in necrotic cell death in prostate cancer cells.

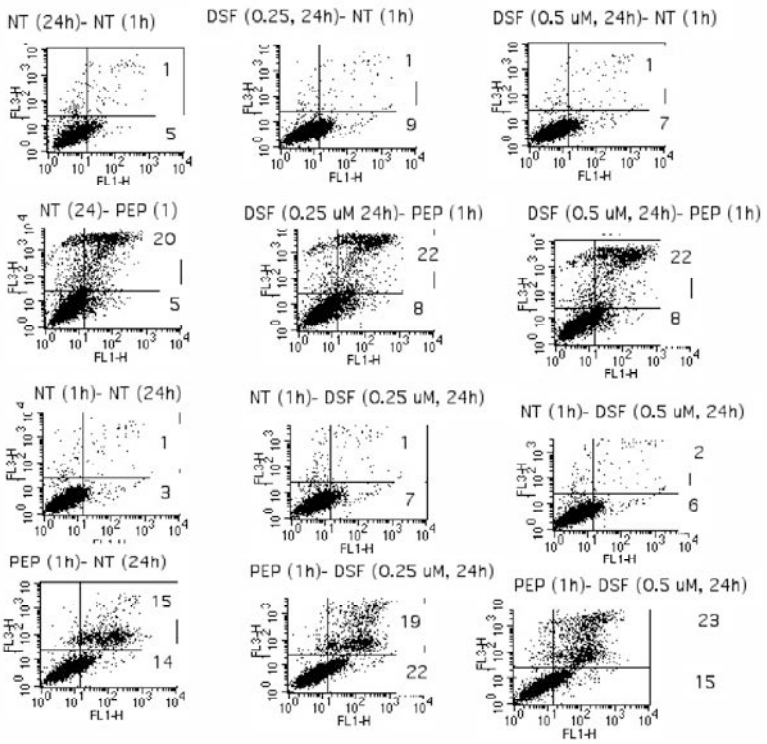
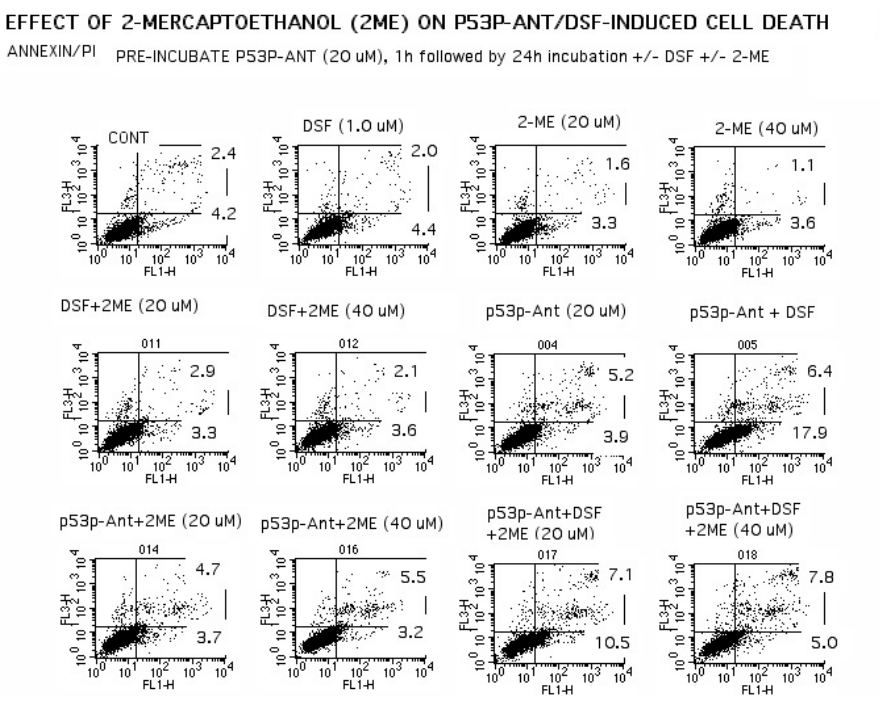


Figure 4: Effect of order of treatment with DSF or p53p-Ant on cell death.

First Row -Either no treatment (NT) or DSF for 24h. Second row- Either no treatment, or DSF for 24h followed by p53p-Ant (PEP) for 1 hour. Third row- No treatment for 1h, followed by either no treatment, or DSF treatment for 24h. Fourth row- p53p-Ant for 1 hour. followed by either by no treatment of DSF for 24h. This demonstrates that if cells were incubated with p53p-Ant first followed by DSF, that increased cell death was observed over p53p-Ant alone, whereas if the order of treatment was reversed there was no additional cell death observed.

Figure 5:



In the first 6 histograms cells received no pre-incubation with p53p-Ant followed by DSF, 2-mercaptoethanol (2-ME) or DSF+2-ME. In the last 6 histograms cells were pre-incubated with 20 uM p53p-Ant followed by DSF (1 uM), or DSF+2-ME. Annexin/PI analysis was performed.This experiment indicates that 2ME treatment counteracts the synergistic effects of p53p-Ant and DSF. This implies an interaction of p53p-Ant and DSF involves a redox reaction.

CHAPTER 2: PAC enhances p53-pAnt-induced necrosis in Prostate Cancer cells.

SOW Aim 1, #11. Justification: Caspases are primary focus of our work to trigger increases targeted necrosis. Although PAC is not a caspase inhibitor, it does influence caspase activity within the cell and may direct caspase activity in triggering cell death.

If there are unknown caspases activated in p53p-Ant induced cell death we decided to examine the effect of pre-treatment of paclitaxel (PAC) on p53p-Ant-induced targeted necrosis in prostate cancer, since 1) PAC is known to activate apoptosis and caspases and 2) the taxanes are the standard of care for hormone resistant prostate cancer treatment. We found that cells pre-incubated with paclitaxel (PAC), synergistic necrosis was observed after subsequent treatment with p53p-Ant, however no synergy was observed if cells were first incubated with p53p-Ant (Fig 6). This directionality of effect is important to consider in the determination of possible mechanism of action. Other experiments leading up to and supporting this observation can be found in Figs 7-9. (See individual figures for detailed analysis).

Paclitaxel (PAC) is a widely used chemotherapeutic drug for treatment of various cancers in clinical oncology. Paclitaxel preferentially binds to beta microtubules at sites distinct from binding sites of other microtubule active drugs (15), and stabilizes microtubules, interfering with the mitotic spindle resulting in the failure of chromosomes to segregate. The mechanism of induction of cell death involves several major signal transduction pathways, including the Fas/caspase-8 and MAPK pathways (16). PAC induced cell death in epithelial sarcoma cell lines was enhanced by Fas ligand (Fas-L)(17).

Besides caspase activation, PAC is also known to induce Fas ligand production (18) and we wondered if this may be a mechanism of its interaction with p53p-Ant, since p53p-Ant is known to interact with the Fas pathway. We found that paclitaxel treatment alone was shown to induce the transcription and translation of Fas ligand in the first 24h, as shown by RT-PCR (Fig 11) and western blotting (Fig 12A), respectively. Gemcitabine (Gemzar), by contrast, did not induce Fas ligand expression (Fig. 12B).

Fas-Associated protein with Death Domain (FADD) is an adaptor molecule that bridges the Fas receptor, and other death receptors, to caspase-8 through its death domain to form the death-inducing signaling complex

(DISC) during apoptosis. Further preliminary studies showed that addition of an adenovirus containing dominant negative FADD developed in our laboratory, a truncated version of FADD lacking the 18 N-terminal amino acids of FADD, enhanced necrosis by p53p-Ant alone (Fig 13), and repressed the synergistic activity with PAC (Fig. 14), suggesting the involvement of FAS/FADD pathway in the increased activation of necrosis (Fig 13). Western analysis showed the expression of DN-FADD (Fig 10).

Previous studies indicated that p53p-Ant activates the Fas receptor by flipping the receptor in the extracellular membrane making it available for activation by ligand (19). Therefore, pre-treatment with paclitaxel may prime the cells by initial induction of Fas ligand so that subsequent exposure to p53p-Ant efficiently activates the Fas/FADD cell death pathway. Our working model is depicted in Fig 16. We are currently testing whether the synergistic cell death between PAC and p53p-Ant is dependent on the induction of Fas ligand by addition of Fas ligand or Fas receptor siRNA.

Figure 6: Effect of pre-incubation with p53p-Ant followed by treatment with paclitaxel (PAC) or pre-incubation with PAC followed by treatment with p53p-Ant.

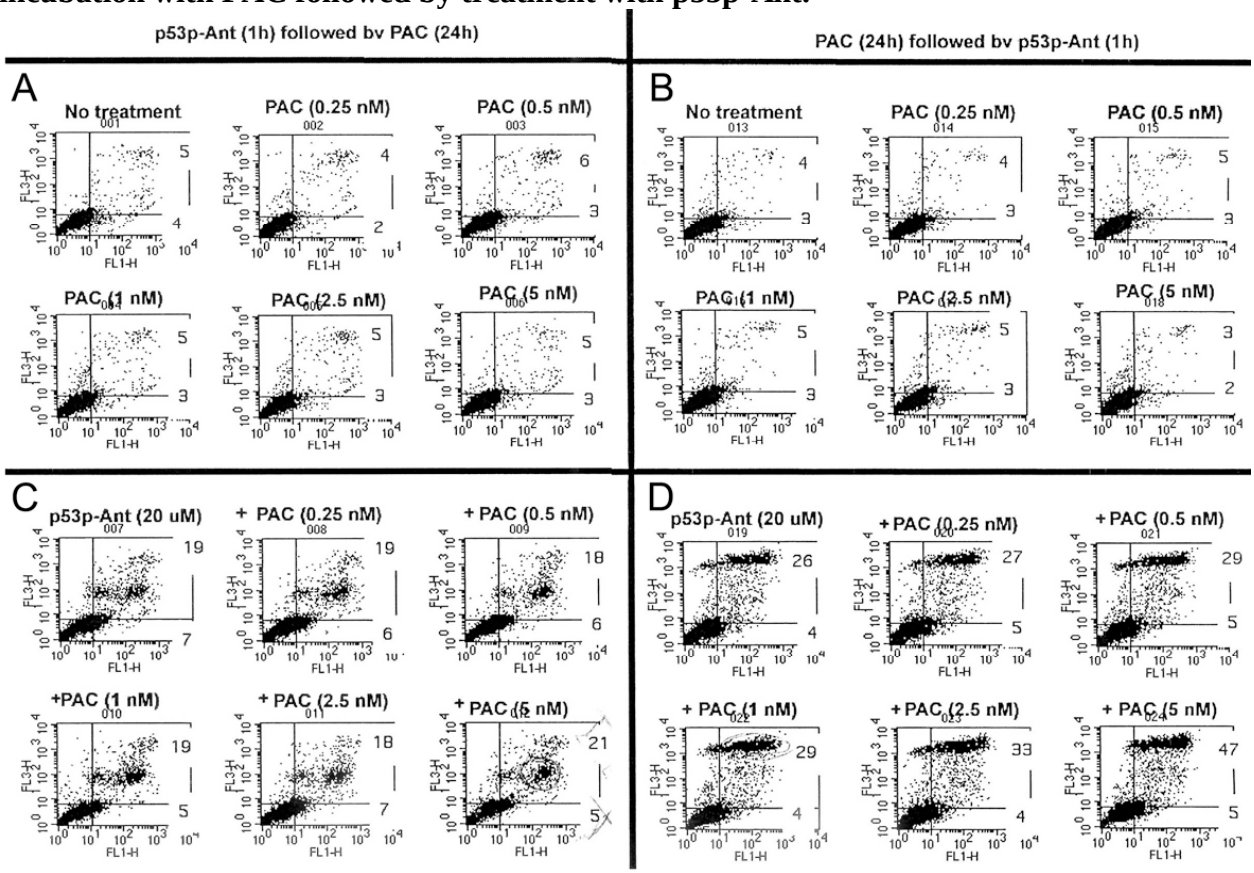
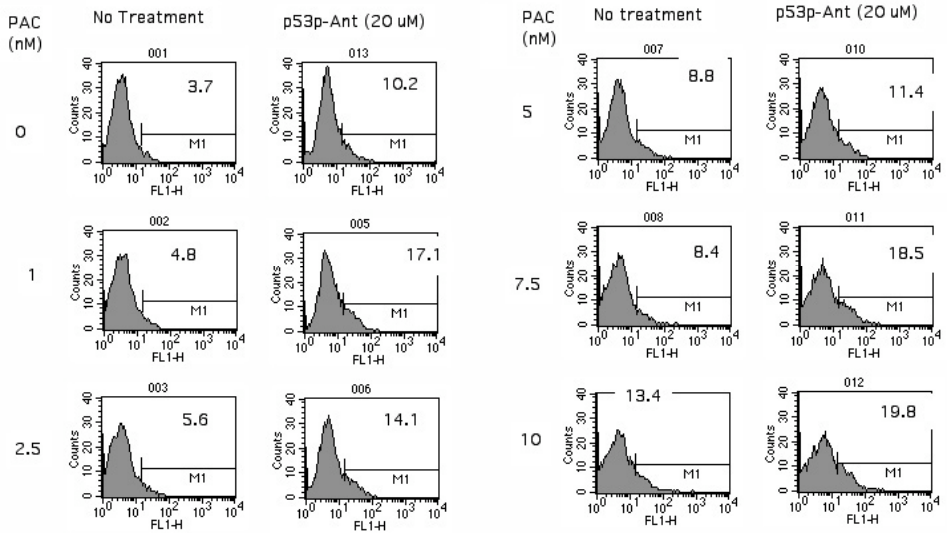


Figure 6 Legend: Section A received no p53p-Ant for 1 h followed by indicated concentrations of PAC for 24h, whereas section C was treated with 20 uM p53p-Ant for 1 h followed by indicated concentrations of PAC. Sections B and D were first treated with PAC as indicated, followed by no p53p-Ant (B) or 20 uM p53p-Ant (D) for 1 hour. All cells were treated for a total of 25h and analyzed with Annexin V/PI staining by cytofluorometry. At least 5000 cells were counted. Data was analyzed using Cell Quest software.

Results indicate that when cells were treated with p53p-Ant they became primarily necrotic and previously determined (upper right quadrant). If p53p-Ant preceded PAC treatment there was no additional cell death observed (C). The percentage of necrotic cells ranged from 19-21%. If PAC treatment preceded p53p-Ant treatment, there was a synergistic cell death observed started at concentrations of PAC equal to 2.5 nM (33%) and 5 nM (47%) (D). The result at 5 nM represents nearly a doubling of necrosis observed with prior incubation with PAC. PAC alone produced no increase in cell death. The results indicate that if PAC treatment preceded p53p-Ant there was synergistic cell death observed but if p53p-Ant treatment preceded PAC there was no synergistic cell death observed.

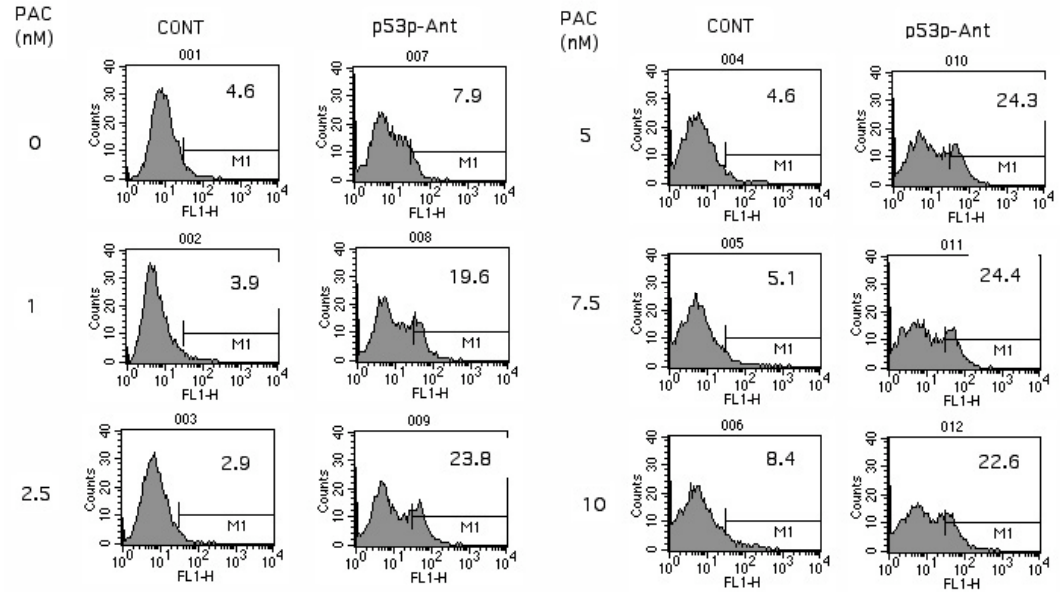
Figure 7: Effect of PAC pre-incubation on p53p-Ant sensitivity in DU-145 cells.



Columns 1, 3 (from left) received concentrations of PAC for 24h as indicated and no p53p-Ant treatment for 1 h.. Columns 2, 4 received received concentrations of PAC for 24h as indicated followed by p53p-Ant (20 uM). For experimental details refer to Figure 1. **Negative Result.** There was synergistic cell death observed when cells were pre-incubated with 1 nM PAC, but none at other concentrations. Problems with experiment were that cells were overly sensitive to PAC at low

concentrations producing a high background and masking the synergistic effect observed. New cells were thawed for further experiments.

Figure 8: Effect of PAC pre-incubation on p53p-Ant sensitivity in DU-145 cells.



Columns 1, 3 (from left) received concentrations of PAC for 24h as indicated and no p53p-Ant treatment for 1 h.. Columns 2, 4 received concentrations of PAC for 24h as indicated followed by p53p-Ant (20 uM).

For experimental details refer to Figure 1.

There was a 1.7 fold increase in necrosis without PAC pre-incubation (4.6-7.9%), whereas with PAC pre-incubation there was a synergistic response that ranged from 5-8 fold, with the highest response observed at 2.5 nM PAC. Thus the sequence of drugs was very important for induction of necrosis when switching from apoptosis.

Figure 9: Effect of PAC pre-incubation on p53p-Ant sensitivity in DU-145 cells.

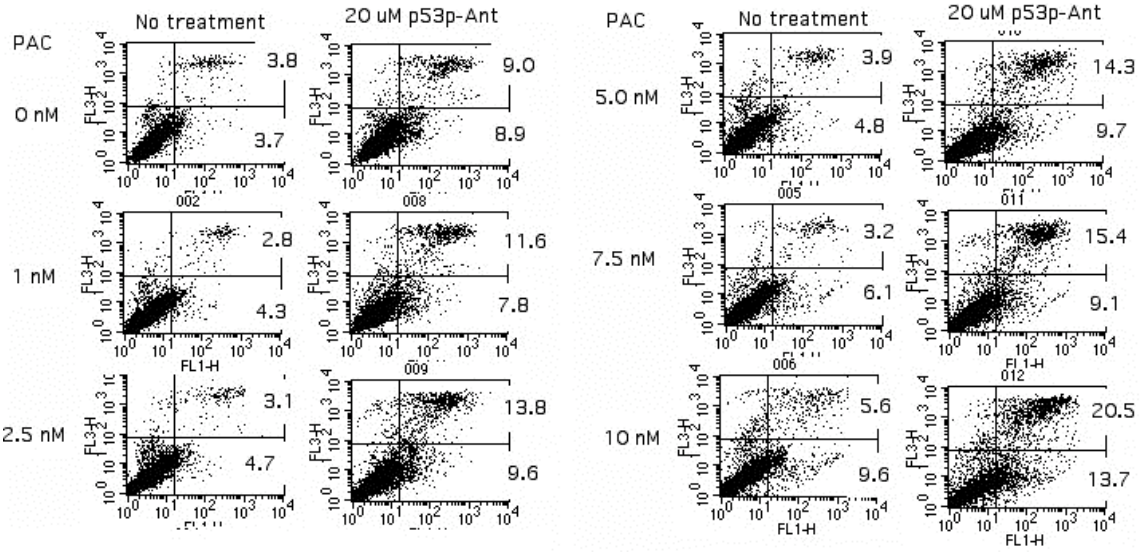
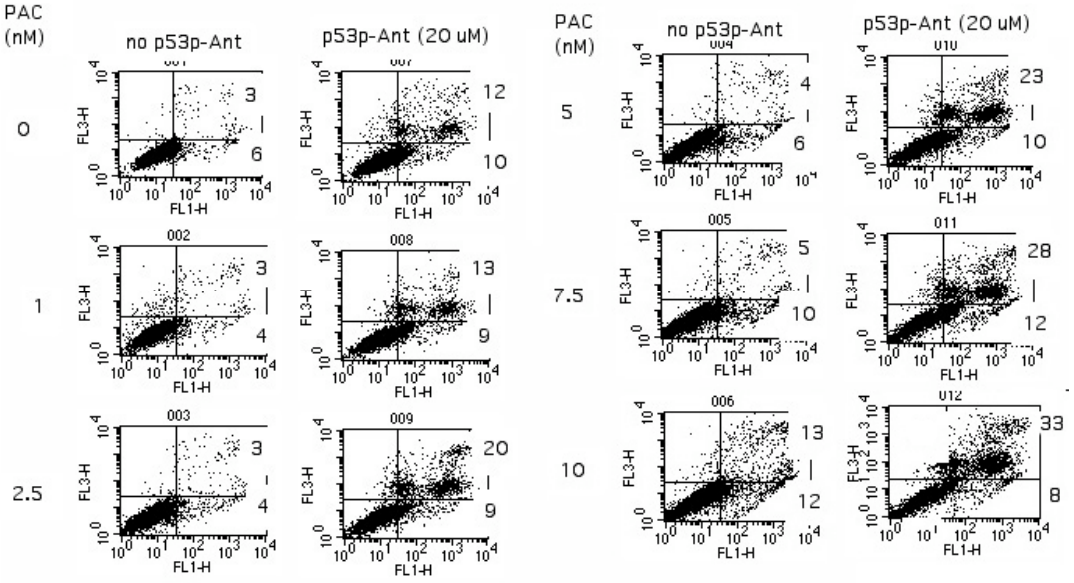


Figure 9 legend: Columns 1, 3 (from left) received concentrations of PAC for 24h as indicated and no p53p-Ant treatment for 1 h.. Columns 2, 4 received concentrations of PAC for 24h as indicated followed by p53p-Ant (20 uM). For experimental details refer to Figure 1. There was a 2.4 fold increase in necrosis without PAC pre-incubation (3.8-9.0%), whereas with PAC pre-incubation there was a synergistic response that ranged from 3.7-4.8 fold, with the highest response observed at 7.5 nM and 2.5 PAC. Therefore, the highest synergistic response was observed with PAC concentrations ranging from 2.5 to 7.5 nM in this experiment.

Figure 10: p53p-Ant-PAC: Effect of p53p-Ant pre-incubation on PAC sensitivity in DU-145 cells.



Columns 1, 3 (from left) received concentrations of PAC for 24h as indicated and no p53p-Ant treatment for 1 h. Columns 2, 4 received p53p-Ant (20 uM) for 1 h followed by PAC for 24h as indicated.

For experimental details refer to Figure 1. As expected there was a large increase in necrotic cells (3-12%) and some increase in apoptotic cells (6-10%) after 1 hour without pretreatment of PAC (cells were incubated with p53p-Ant and then analyzed 24h later). If p53p-Ant incubation was followed with PAC, there was some enhancement of cell death over no PAC preincubation: at 1 nM PAC it was 1.1 fold, at 2.5 nM 1.9 fold, at 5 nM 2.1 fold, at 7.5 nM 2.6 fold and at 10 nM 2.2 fold. Therefore, there was a clear advantage for initial incubation with PAC followed by p53p-Ant rather than the reverse as shown here.

Figure 11: RT-PCR showing Fas ligand expression 24 hour after paclitaxel (PAC) treatment

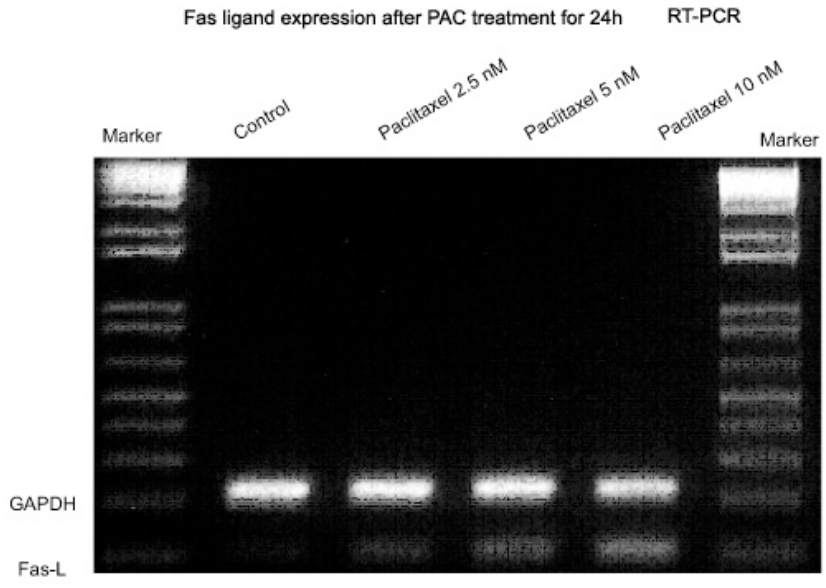


Figure 12A

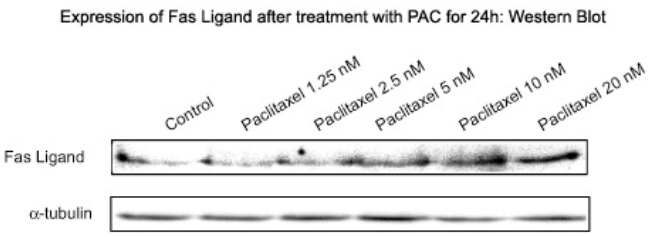
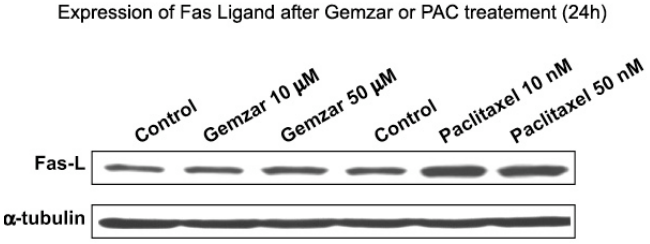
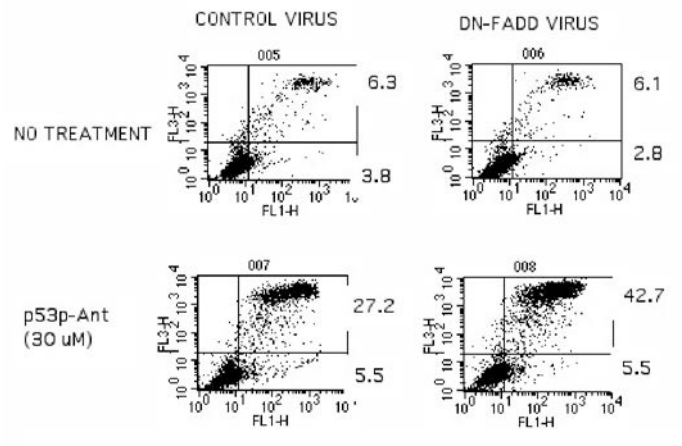


Figure 12B



7A) Western blot analysis of Fas ligand expression after incubation with various concentrations of paclitaxel or 7B, Paclitaxel and Gemzar (gencitabine) after 24h. This means that the increase of FasL is specific for PAC, a prototypic taxane, and not induced by Gemcitabine, a prototypic nucleoside analogue.

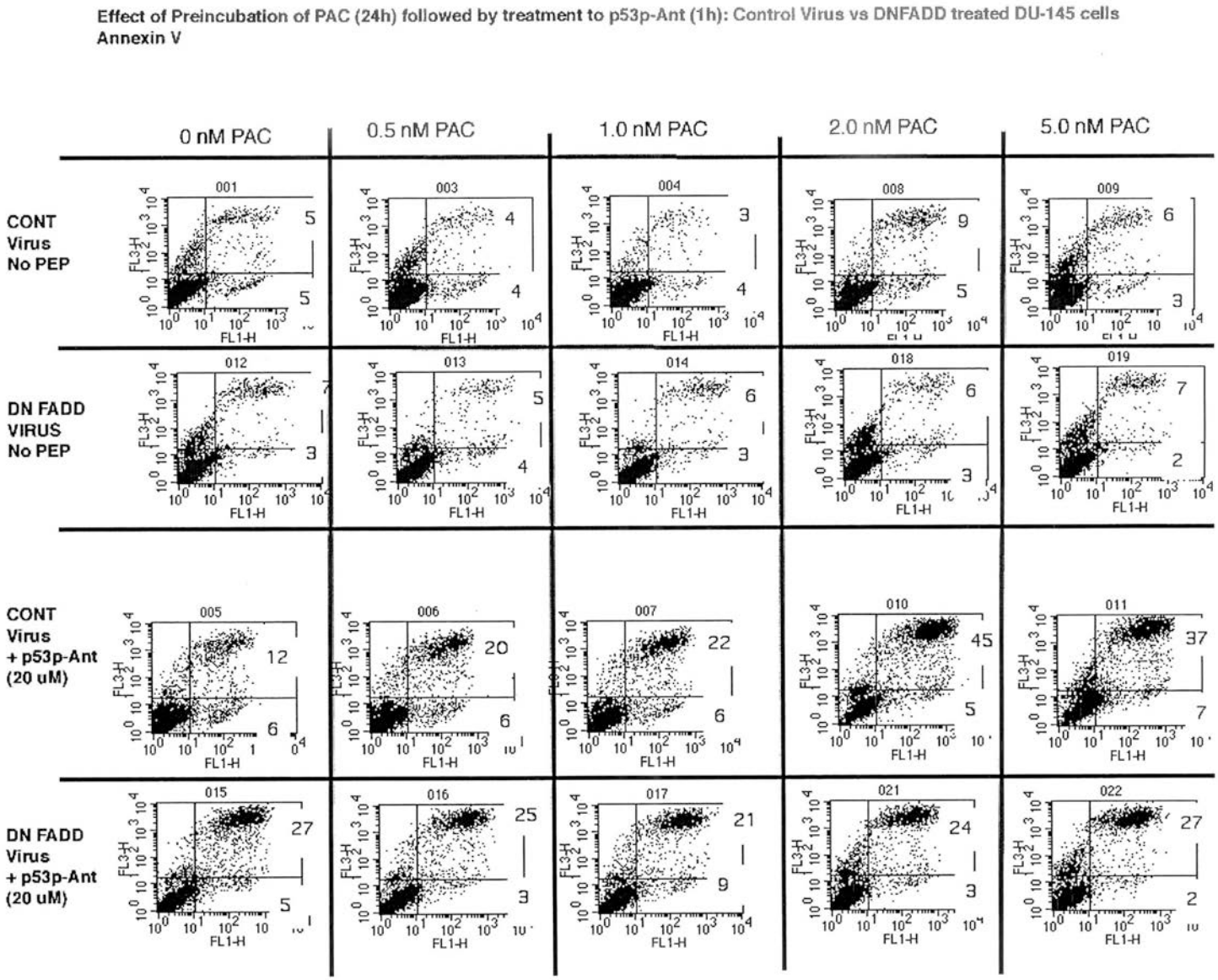
Figure 13:



Treatment with DN FADD enhanced p53p-Ant-induced necrosis. As shown above, if cells treated with the control virus were exposed to p53p-Ant, there was 27.2% necrosis observed. If cells were treated with DN FADD virus, p53p-Ant exposure resulted in 42.7% necrotic cells representing a 15.5% increase in necrosis. There was no increase in necrosis is cell were treated with DN FADD virus alone over control virus.

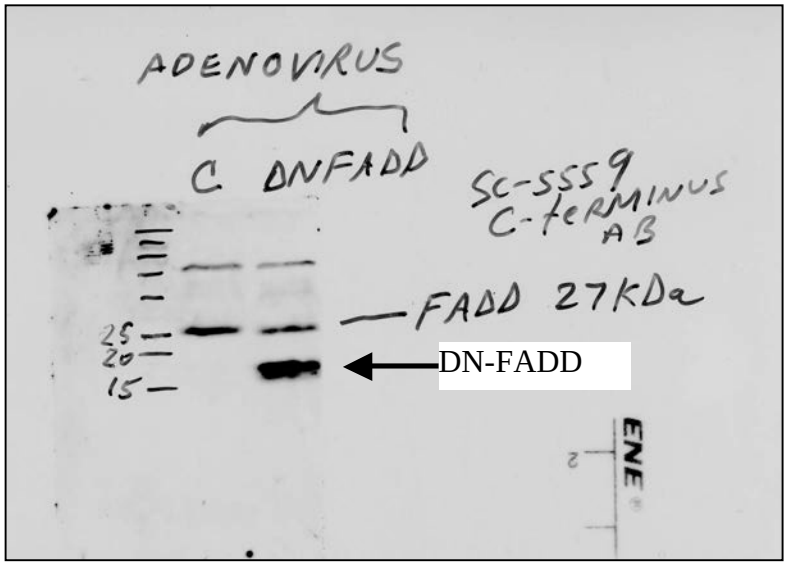
Annexin/PI Analysis: For Anx V/ PI analysis, cells were incubated with Alexa Fluor 488 (Molecular Probes, Eugene OR) for 5 min followed by 0.33 μg/ml PI for 1 min. Five thousand cells were analyzed immediately by FACS (channels FL-1 for Anx V and FL-3 for PI). ***shRNA DN-FADD* constructs:** pAd/U6/p53-SiRNA and pAd/U6/shuffled-p53-SiRNA were inserted into an E1/E3-deleted adenovirus vector and propagated in 293A cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The virus particle titer after filtration-based concentration (Adeno-X Virus Purification Kit, Clonetech Laboratories, Inc.) was 1 x 10^9 pu/ml as determined by plaque titration assay in 293A cells. After the second round of infection, the cells were split, allowed to adhere overnight and exposed to p53p-Ant for 3h and Annexin V (Anx V) /PI analysis performed.

Figure 14: Effect of Pre-incubation of PAC (24h) followed by treatment with p53p-Ant (1h) in Prostate cancer cells exposed to Control virus or DN-FADD Virus.



As shown above, treatment with PAC alone (up to 5 nM) had little effect of DU-145 cell death in the absence of p53p-Ant. Addition of p53p-Ant alone resulted in 12 and 27% necrotic cells with control and DN FADD virus addition. However, if cells were pretreated with PAC there was a synergistic response in cells treated with Control virus, wich was not observed in cells treated with DN-FADD virus. Therefore, the preliminary conclusion is that although DN-FADD increased cell death with p53p-Ant alone, the synergistic response between p53p-Ant and PAC is inhibited by DN-FADD. Further experiments are required to confirm and expand upon this result. For experimental details see Figure 8.

Figure 15: Western blot analysis showing expression of DN-FADD and wt FADD.



Western blot analysis confirming expression of DN-FADD shRNA after treatment with virus for 36h. Freshly split DU-145 cells were treated with 50 MOI virus particles. *shRNA* constructs pAd/U6/p53-SiRNA and pAd/U6/shuffled-p53-SiRNA were inserted into an E1/E3-deleted adenovirus vector and propagated in 293A cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The virus particle titer after filtration-based concentration (Adeno-X Virus Purification Kit, Clonetech Laboratories, Inc.) was 1 x 10^9 pu/ml as determined by plaque titration assay in 293A cells. DU-145 cells in log phase were infected in two successive 48h periods with pAd/U6/p53-ShRNA or pAd/U6/shuffled-p53-ShRNA at 25 MOI. After the second round of infection, the cells were split, allowed to adhere overnight and exposed to p53p-Ant for 6h and Annexin V (Anx V) analysis performed (see below). This result indicated that DN-FADD is highly expressed in cells treated with the DN-FADD containing virus.

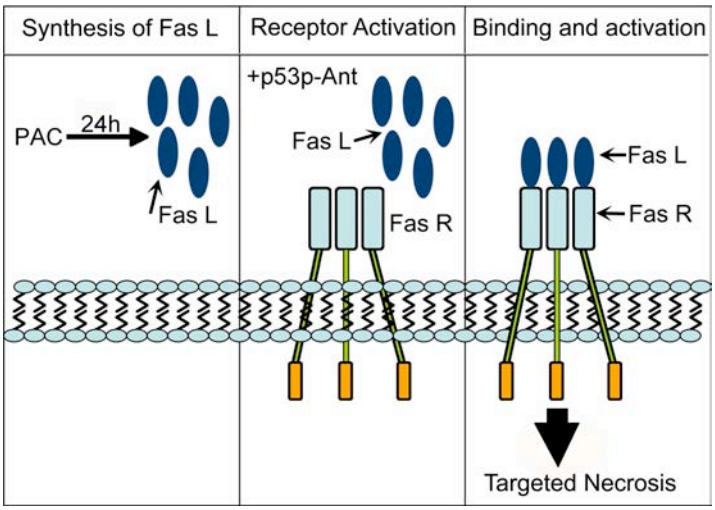


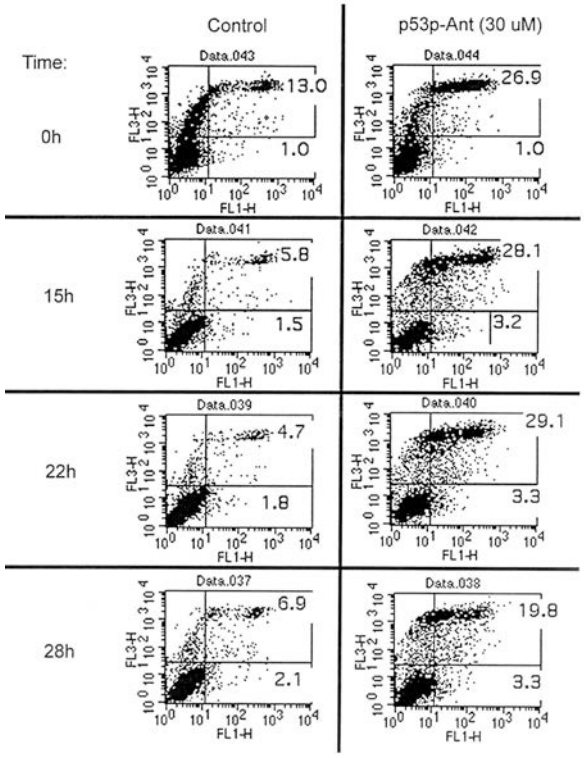
Figure 16: Mechanistic model for increasing necrosis in prostate cancer cells by pre-treatment with PAC.

Panel 1: Pretreatment with PAC results in expression of Fas ligand (Figure 6 and 7). This process requires 24 h time for expression of adequate amounts of Fas Ligand. Panel 2: Exposure of the cells to p53p-Ant results in activation of the Fas receptor and exposure to the external surface of the cell, making the receptor accessible to engagement by Fas ligand. Panel 3: This results in targeted necrosis in prostate cancer cells. If the exposure of DU-145 cells to p53p-Ant and PAC is reversed, there is no increased activation of targeted necrosis because either the Fas ligand is not available at the correct time, or receptors are not available at the correct time for activation.

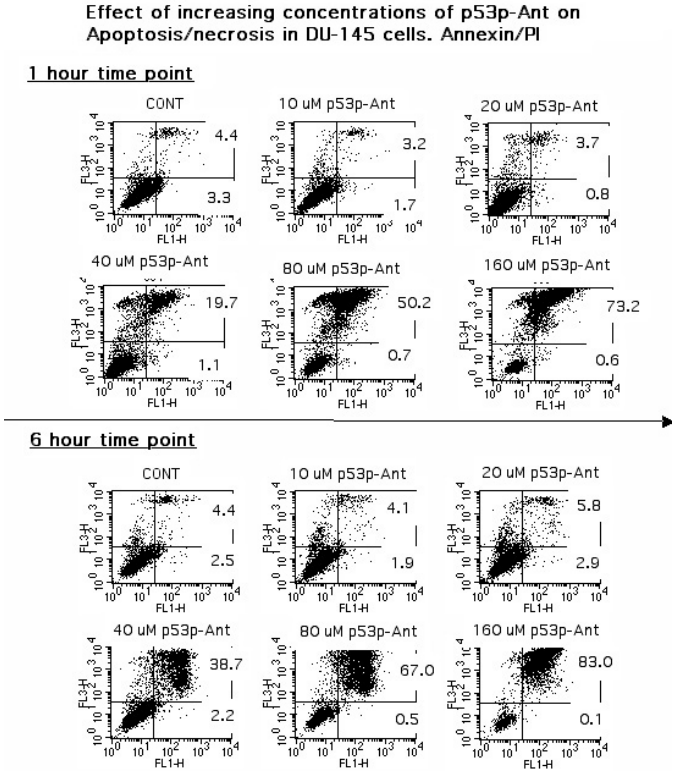
CHAPTER 3: Initial Studies with Knockdown of Cyclophilin D, Cathepsins and the nature of Targeted Necrosis. SOW: AIM 1, #6 #8

Fig. 17: Experiments performed to if concentration or pre-plating times to determine under what conditions cells undergo necrosis and whether apoptosis can be induced under different plating conditions or p53p-Ant concentrations. The results indicate that necrosis was always observed and it seemed to be an all or nothing phenomena rather than showing intermediate transitions between apoptosis and necrosis. This suggests that there may be a specific cell death pathway that is specific for p5p-Ant-induced necrosis in these cells.

A



B



C

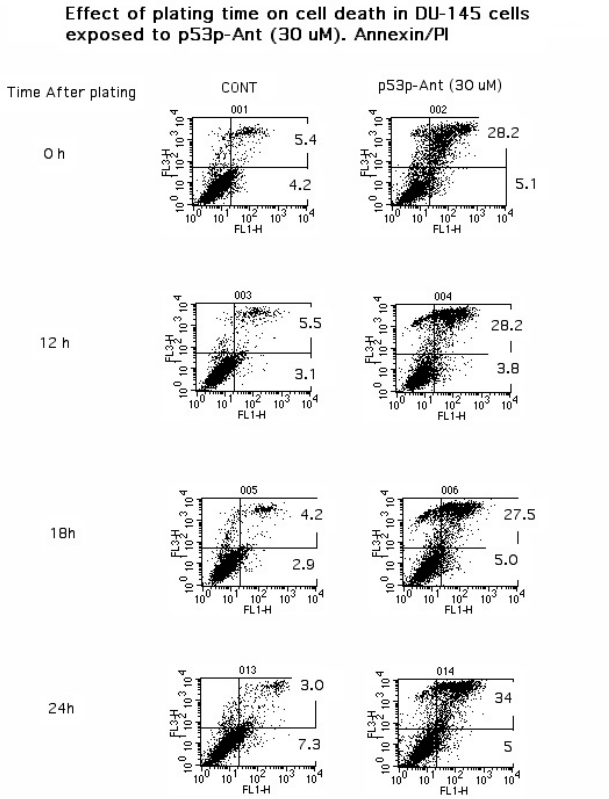
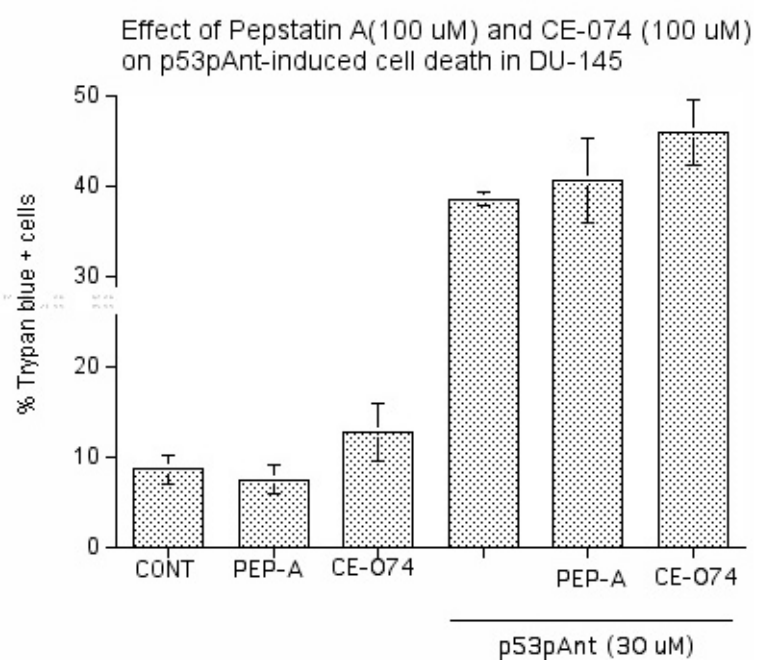


Fig. 18: Effect of cathepsin inhibitors on p53p-Ant-induced cell death. PepstatinA is an aspartic protease inhibitor and and inhibitor or cathepsin D and E. CE-074 is an inhibitor of cathepsin B. The results suggest that cathepsins B, D and E are not involved in p53p-Ant induced cell death in prostate cancer cells.



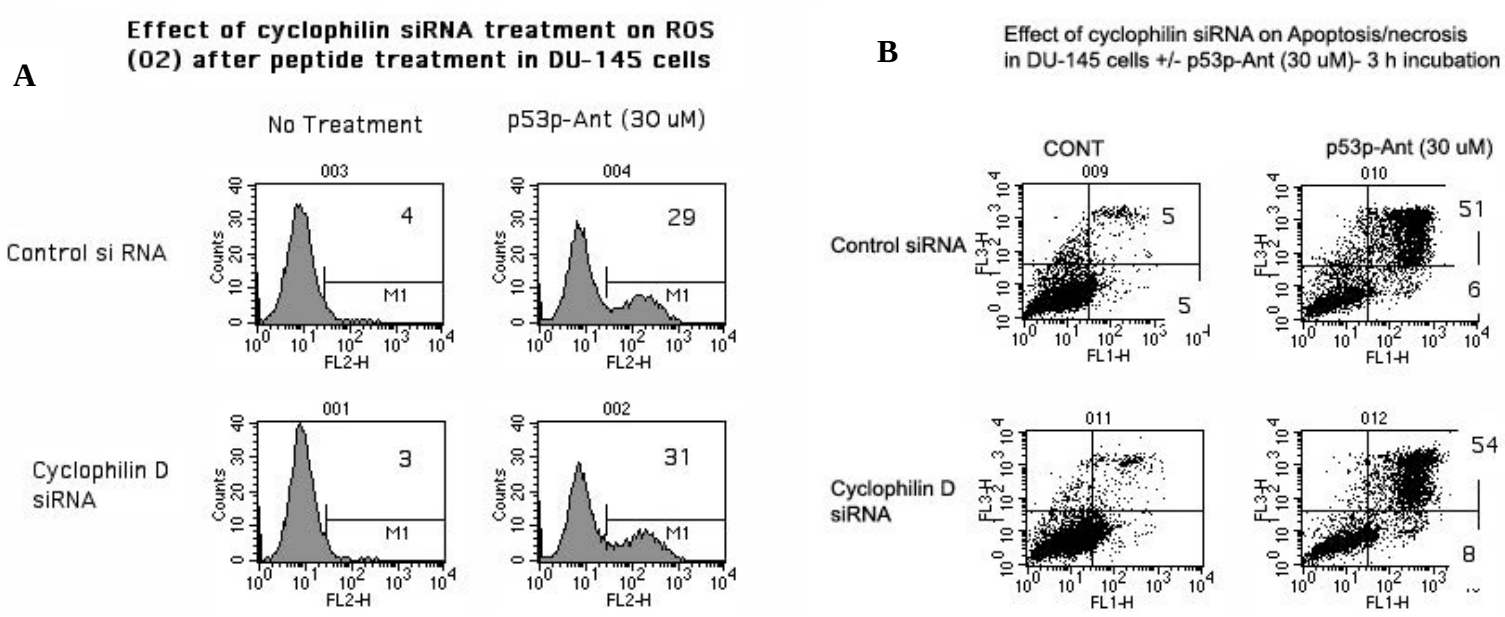


Fig. 19: Preliminary results above show that cyclophilin D siRNA treatment does not affect p53p-Ant induced cell death. However, western analysis is required to determine whether cyclophilin D protein levels were affected sufficiently to be able to rule out the effect of cyclophilin D in p53p-Ant induced cell death.

Key Research Accomplishments:

- Synergistic effect found with a widely used taxane chemotherapy drug, paclitaxel, and p53p-Ant in induction of targeted necrosis, leading to practical clinical application
- Mechanistic understanding of this synergistic effect, dependent on FAS/FADD pathway, which may lead to wider application to cancers that do not undergo targeted necrosis
- Synergistic effects found with widely used and clinically safe drug, disulfiram, leading to potential new chemotherapy regimen together with p53p-Ant

Reportable Outcomes:

- Manuscript in preparation: **Synergistic targeted necrosis with a p53 C-terminal peptide and the taxane Paclitaxel for the treatment of human hormone resistant prostate cancer cells.**

Conclusions:

By understanding the mechanisms and pathways of targeted necrosis, as it differs from classical apoptosis, may help to elucidate how we can manipulate available drugs and p53 peptides for inducing this alternate pathway in human prostate cancer cells to circumvent resistance to classical apoptosis. This may lead to a novel therapeutic approach, which overcomes the resistance to apoptosis which occurs in prostate cancer when exposed to taxanes which are the major chemotherapy drug class useful against this cancer. Animal studies using p53p-Ant in combination with DSF and PAC as this report describes, as fulfillment of Aims 2 and 3 will begin shortly.

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